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RM PTO-1390 (REV. 1-98) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	
230-148P	
U.S. APPLICATION NO. (If known, see 37 CFR 1.5)	
09/509945	

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/JP99/04167	August 3, 1999	August 4, 1998

TITLE OF INVENTION

MUTANT BARNASE GENE AND TRANSGENIC PLANT TRANSFORMED BY SAID GENE

APPLICANT(S) FOR DO/EO/US

HAMADA, Kazuyuki; NAKAKIDO, Fumio

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39 (1).
4. ☐ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ has been transmitted by the International Bureau.
   c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(3)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(2)).
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

1. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.-1449 and International Search Report (PCT/ISA/210)
2. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
3. ☒ A FIRST preliminary amendment.
   ☐ A SECOND or SUBSEQUENT preliminary amendment.
4. ☐ A substitute specification.
5. ☐ A change of power of attorney and/or address letter.
6. ☒ Other items or information:
   1.) PCT Request (PCT/RO/101)
   2.) Sequence Listing (12 pages)
   3.) Verification of Translation
   4.) Zero (0) sheets of Formal Drawings

09/744745	PCT/JP99/04167	ATTORNEY'S DOCKET NUMBER 230-148P

The following fees are submitted:	CALCULATIONS	PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$970.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$840.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$690.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$670.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$96.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 840.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 0	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	30 - 20 =	10	X **$18.00**	$ 180.00	
Independent Claims	5 - 3 =	2	X **$78.00**	$ 156.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)		Yes	+ **$260.00**	$ 260.00	
			TOTAL OF ABOVE CALCULATIONS =	$ 1436.00	
Reduction of ½ for filing by small entity, if applicable. Verified Small Entity statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).				$ 0	
			SUBTOTAL =	$ 1436.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐20 ☐30 months from the earliest claimed priority date (37 CFR 1.492(f)).			+	$ 0	
			TOTAL NATIONAL FEE =	$ 1436.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property			+	$ 40.00	
			TOTAL FEES ENCLOSED =	$ 1476.00	
				Amount to be: refunded	$
				charged	$

a. ☒ A check in the amount of $ **1476.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account. No. _____ in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 02-2448.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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PATENT
230-148P

IN THE U.S. PATENT AND TRADEMARK OFFICE

Applicant:	HAMADA, Kazuyuki et al.		
Int'l. Appl. No.:	PCT/JP99/04167		
Appl. No.:	New	Group:	
Filed:	April 4, 2000	Examiner:	
For:	MUTANT BARNASE GENE AND TRANSGENIC PLANT TRANSFORMED BY SAID GENE		

PRELIMINARY AMENDMENT

BOX PATENT APPLICATION
Assistant Commissioner for Patents
Washington, DC 20231

April 4, 2000

Sir:

The following Preliminary Amendments and Remarks are respectfully submitted in connection with the above-identified application.

AMENDMENTS

IN THE SPECIFICATION:

Please amend the specification as follows:

Before line 1, insert --This application is the national phase under 35 U.S.C. § 371 of PCT International Application No. PCT/JP99/04167 which has an International filing date of August 3, 1999, which designated the United States of America.--

REMARKS

The specification has been amended to provide a cross-reference to the previously filed International Application.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

By _____ #32,868
for Gerald M. Murphy, Jr., #28,977

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(Rev. 01/08/2000)

SPECIFICATION

MUTANT BARNASE GENE AND TRANSGENIC PLANT TRANSFORMED BY SAID GENE

The present application claims priority from Japanese Patent Application No.: Hei 10-220060, the disclosure of which is incorporated herein by reference.

FIELD OF THE INVENTION

This invention relates to a mutant barnase gene which makes it possible to efficiently yield a male sterile transgenic plant when expressed in a specific site of a plant, in particular, anther-specifically. The present invention further relates to a recombinant vector capable of expressing the mutant barnase gene of the invention in host cells, a plant transformed by this vector and a method for constructing a transgenic plant.

PRIOR ART

Barnase is an RNase originating in *Bacillus amyloliquefaciens* (S. Nishimura and M. Nomura, Biochem. Biophys. Acta 30, 430-431:1958; R.W. Hartley, J. Mol. Biol., 202, 913-915:1988). This enzyme has 110 amino acid residues and hydrolyzes RNA. When expressed in cells, this enzyme inhibits the functions of the cells as a result of its potent RNase activity and thus causes cell death in many cases. By using this characteristic, it is therefore expected that the function of the specific site can be selectively controlled by expressing the barnase gene in a specific site of a plant.

PCT International Publication WO89/10396 discloses a

technique whereby a male sterile plant is obtained by constructing a male sterility gene by ligating the above-described barnase gene to the downstream of an anther tapetal cell-specific expression promoter and introducing the thus obtained gene into a plant. Male sterilization techniques are of great value in efficiently developing an F1 hybrid variety.

When the barnase gene is employed as a male sterility gene, however, it is frequently observed that resulting male sterile transgenic plants exhibit unfavorable characteristics. PCT International Publication WO96/26283 refers to this problem in rice. It is also reported that similar phenomena are observed not only in rice but in lettuce (Scientia Horticulturae 55, 125-139:1993; Arlette Reymaerts, Hilde Van de Wiele, Greta De Sutter, Jan Janssens: Engineered genes for fertility control and their application in hybrid seed production). According to this report, a plant with depressed activity was constructed by introducing a male sterility gene comprising a tobacco anther-specific promoter (TA29) and a barnase gene into lettuce.

Although reasons for these phenomena have not been accurately clarified so far, it is assumed that the so-called "position effect" of the site in which the gene is transferred may account for the mechanism (PCT International Publication WO96/26283). More specifically, a desired male sterile plant should be constructed if the male sterility gene is expressed exclusively in the target site (i.e., anthers). However, there is a possibility that the barnase

might be expressed also in tissues other than anthers although in a very small dose under the action of expression regulators (for example, an endogenous enhancer) existing in the vicinity of the gene transfer site. In such a case, the unfavorable characteristics as described above can appear because barnase has a strong enzymatic activity.

To overcome this problem, the method disclosed in PCT International Publication WO96/26283 exploits the character of cauliflower mosaic virus 35S promoter (hereinafter referred to as CaMV35S promoter) of being expressed potently in tissues other than anthers. Namely, barstar, i.e., an inhibitory protein against barnase is employed therein. The barstar gene ligated to a CaMV35S promoter is transferred into a plant simultaneously with a barnase gene and then the barstar gene is constitutively expressed in tissues other than the anthers, thereby eliminating the effects of the barnase in tissues other than the anthers. However, it is necessary in this method to transfer not only a barnase gene but also a barstar gene, and hence, the problem is that the application of this technique to the breeding of F1 varieties of rice or corn seeds may give rise to a gene silencing. "Gene silencing" is a phenomenon wherein the expression of a gene is inhibited when plural copies of the foreign gene are introduced. It is known that this problem frequently occurs when the expression of a foreign gene is effected by a 35S promoter, though the detailed mechanism thereof has not been clarified yet (R.B. Flavel, Proc. Natl. Acad. Sci. USA 91, 3490-3496:1994; J. Finnegan, Bio.

Technology 12, 883-888:1994; M.A. Matzke and A.J.M. Matzke, Plant Physiol., 107, 679-685:1995). Since, in rice and corn, a barstar gene is used as a "fertility-restoring gene" in the pollen parent (father) so as to allow the pollen in the F1 generation to restore the fertility (C. Mariani, et al., Nature 357, 384-387:1992), if an MS plant (mother) is constructed by the method with the use of a barstar gene as reported in WO96/26283, the F1 plant has plural copies of the barstar gene. In such a case, there is a possibility that the expression of the gene may be inhibited due to the gene silencing.

SUMMARY OF THE INVENTION

The present invention provides a method for constructing a male sterile plant by using a barnase gene without resort to a barstar gene.

The present invention further provides a mutant barnase gene to be used in the above method and a process for producing the same.

DETAILED DESCRIPTION OF THE INVENTION

In the present invention, the DNA sequence of barnase gene (R.W. Hartley, J. Mol. Biol. 202, 913-915:1988) is mutated at least in part and then the thus obtained mutant barnase gene is anther-specifically expressed in a plant so as to make the plant substantially male sterile without any substantially disadvantageous effect on the tissues other than anthers.

The mutation can be performed by a known method such as site-specific mutagenesis, deletion of a fragment by

using restriction enzymes or the low fidelity PCR method. Among all, it is preferred to use the low fidelity PCR method. This method is described in detail in D. Leung, E. Chen and D. Goedda, Technique 1, 11-15:1989; Y.Z. Xiaoping and R.H. Ebright, Nucleic Acid Res. 19, 6052:1991; G.C. Rice et al., Proc. Natl. Acad. Sci. USA 89, 5467-5471: 1992 and the contents of these documents are incorporated herein by reference. By using this technique, PCR is carried out under such conditions as to induce some errors during the amplification reaction. Thus, random mutations can be introduced into the target DNA fragment.

Primers to be used in the low fidelity PCR method in the present invention are selected as in the conventional PCR method. It is preferred that each of these primers has a similar number of nucleotides as in the conventional PCR method.

The present inventors performed PCR by using a DNA containing the sequence represented by SEQ ID NO:1 as a template with the use of a combination of primer 1 (5'-CGTTCGGCTC GATGGTACCG GTTATCAACA CGTTTGA-3': SEQ ID NO:6) and primer 2 (5'-CCTCTAGATT ATCTGATTTT TGTAAAGGTC TGATAATG-3': SEQ ID NO:7) under such conditions as to induce errors. Thus, a mutant barnase gene having the DNA sequence represented by SEQ ID NO:3 was isolated. The sequence represented by SEQ ID NO:1 employed herein is the sequence of the coding region of the barnase gene contained in the known plasmid pVE108 (WO92/13956). This sequence corresponds to the wild type barnase gene from which the

unnecessary moiety corresponding to the secretion signal in the N-terminal side has been deleted.

It is also possible to acquire different mutant barnase genes by a similar method.

The PCR amplification product is cloned in a host (for example, *Escherichia coli*) and clones containing a mutant barnase gene is isolated. In this cloning, clones having a mutant barnase gene may be screened by assaying the RNase activity expressed by the gene. However, it is advantageous to screen the clone by the method consisting of the following two steps by taking advantage of the fact that the RNase activity of barnase affects the growth of *E. coli*.

In the first step, a plasmid is prepared by using the mutant barnase gene obtained above and then *E. coli* is transformed thereby. The growth of the *E. coli* transformant thus obtained will be inhibited by the activity of the mutant barnase. Based on this fact, a colony growing slowly (i.e., a small colony), compared with *E. coli* having a control vector free from any barnase gene, is selected. It is expected that the thus selected *E. coli* strain will contain the mutant barnase gene. To confirm that the inhibition of the growth of *E. coli* is due to the expression of the mutant barnase gene, the second step is then carried out.

In the second step, a barstar gene is employed. As described above, barstar is an inhibitory protein against barnase. In *E. coli* which expresses barstar, the enzymatic activity of barnase is inhibited and thus the degradation of

mRNA, which otherwise takes place in the presence of barnase, can be inhibited. Therefor if, *E. coli* which expresses a barstar gene is transformed with a barnase gene, the growth will not be inhibited. Accordingly, it is expected that the thus obtained *E. coli* transformant will produce little difference in growth rate from the transformant constructed by the control plasmid which is free from any barnase gene and, therefore, these transformants will produce little difference in colony size too. Based on this principle, a plasmid is prepared from the *E. coli* strain selected in the first step. The plasmid is then used to transform another *E. coli* strain, in which the barstar gene is constitutively expressed. Thus, mutant barnase gene-containing colonies having almost the same size as the one of the *E. coli* transformed by the control plasmid are selected. The *E. coli* with the constitutive expression of the barstar gene to be used herein can be prepared by, for example, the method as will be described in Example 1 hereinafter.

The DNA sequence of the thus obtained mutant barnase gene may be analyzed by a conventional method, if necessary, to thereby examine the mutation in detail.

A preferred example of the mutant barnase gene of the present invention is one which has the DNA sequence represented by SEQ ID NO:3. Compared with the nucleotide sequence of SEQ ID NO:1 coding for the wild type activity, the nucleotide sequence encoding this gene has an insertion of "T" at the 15-position from A in the initiation codon ATG and a deletion of "A" at the 333-position. According to the

normal translation manner, translation will be initiated from ATG at the 1-position of this DNA sequence represented by SEQ ID NO:3, and terminated at the ninth codon which has been converted to a termination codon as a result of the insertion of the "T" at the 15-position. It is unlikely that the thus formed oligopeptide consisting of 8 amino acids has the barnase activity. Further, there is no other ATG or GTG codon from which the translation can be initiated in the correct frame in the vicinity thereof. However, the fact that the growth rate of *E. coli* was restored by the barstar protein strongly suggests that the translation of the barnase protein from the gene of SEQ ID NO:3 proceeded in the correct frame. This is seemingly due to a phenomenon called "frame shift re-coding" whereby the ribosome controls the translation while automatically shifting the reading frame during the course of translation into the protein. This phenomenon, which has been reported in several genes of viruses, *E. coli* and animals, generally depends exclusively on an individual nucleotide sequence and neither any special protein nor translation device is required. The frame shifting efficiency during the translation varies from gene to gene, i.e., ranging from several % to 50%. In the case of the mutant barnase gene, it is considered that the efficiency of the shifting of the reading frame during the translation is not high and thus the translation product in the correct frame is formed in a smaller amount, thereby lowering the activity of the gene.

Moreover, there is a high possibility that other

mutant barnase genes of which the translation efficiency is lowered due to the shifting of the reading frame, as in the case of the DNA sequence represented by SEQ ID NO:3, or some other reasons can be obtained by mutating the barnase gene by, for example, the low fidelity PCR method. Furthermore, there is a high possibility that a gene having a DNA sequence derived from the DNA sequence represented by SEQ ID NO:3 by substitution, deletion, insertion or addition of one or more nucleotides will be a mutant barnase gene which provides a lowered translation efficiency due to the shifting of the reading frame as a result of the insertion of T at the 15-position from A in the initiation codon ATG and/or the deletion of A at the 333-position, as in the DNA sequence represented by SEQ ID NO:3. It is considered that each of these genes, likewise the gene of SEQ ID NO:3, is capable of making a plant substantially male sterile when it is anther-specifically expressed in the plant. Accordingly, these genes are also included in the scope of the present invention similar to the gene of SEQ ID NO:3.

The plant which is to be male sterilized by the mutant barnase gene is not particularly limited, so long as the gene can be transferred into the plant to provide a male sterilized plant. Examples of the plants include rice, corn, tobacco, lettuce and Brassica. Among all, rice and corn are preferred.

In order to male sterilize a plant, the mutant barnase gene is expressed specifically in the anthers of the plant to inhibit the anther functions by the RNase activity of

barnase. Such an anther-specific expression can be achieved by using the method described in WO92/13957. In brief, the mutant barnase gene is ligated to the downstream of an anther-specific promoter and then incorporated into plant cells with the use of an expression vector.

The incorporation may be performed by using the Agrobacterium method, the electroporation method, the particle gun method, etc.

From the transformed plant cells, a complete plant can be formed by the regeneration from a callus of the transformed plant cells in accordance with, for example, the method reported in Y. Hiel et al., Plant J. 6, 271-282:1994.

The male sterility of the thus constructed plant can be confirmed by the inability of the plant to fertilize unless it is pollinated from another fertilizable plant.

Example 1: Preparation of mutant barnase gene

Low fidelity PCR

Primer 1 (CGTTCGGCTC GATGGTACCG GTTATCAACA CGTTTGA: SEQ ID NO:6) and Primer 2 (CCTCTAGATT ATCTGATTTT TGTAAAGGTC TGATAATG: SEQ ID NO:7) were synthesized by a DNA synthesizer (manufactured by Applied Biosystems) in accordance with the method described in S.L. Beaucage et al., Tetrahedron Lett., 22. 1859-1862:1982. Known plasmid pVE108 (WO92/13956) was used as a template and PCR was carried out with the use of the combination of Primers 1 and 2. SEQ ID NO:1 shows the sequence of the coding region of the barnase gene which is contained in pVE108. The reaction was performed for 50 cycles with each cycle consisting of 1 minute at 94°C,

1 minute at 57°C and 1 minute at 72°C in 10 mM Tris-HCl (pH 9.5), 50 mM KCl, 2 mM $MgCl_2$, and 1 mM each of dNTP, 10 ng of the template DNA and 0.5 U of Taq DNA polymerase.

After the completion of the reaction, the amplification product was separated by electrophoreses on agarose gel (2% SeaKem GTG agarose, 1xTAE) and purified by the DEAE-cellulose method (M. Muramatsu, Labo-manual Idenshikogaku (Gene Engineering Labo-Manual), Maruzen, pp.111:1988). By using the purified product thus obtained as a template, PCR was performed again under the conditions as defined above.

Ligation of mutated barnase gene fragment into plasmid vector

The reaction product from the above step was digested with SacI and XbaI in a conventional manner and then purified by agarose gel electrophoresis to give an "insertion fragment". A plasmid to be used for transferring this insertion fragment into *E. coli* can be appropriately selected. For example, use may be made of plasmid pHM1 therefor. The plasmid pHM1 was constructed as follows. Plasmid pBR322 was cleaved at the EcoRI site and blunt-ended by using T4 DNA polymerase (manufactured by Takara Shuzo). Separately, lacZ expression cassette (322 bp) was excised from pUC18 with PvuII and the ends thereof were blunted. The cassette was integrated into the restriction site of the blunt-ended plasmid pBR 322 to give plasmid pHM1.

Plasmid pHM1 was digested with SacI and XbaI followed by dephosphorylation with calf intestine alkaline phosphatase (manufactured by Takara Shuzo) to give a

restriction enzyme-treated plasmid fragment. Into this restriction enzyme-treated plasmid pHM1, the above "insertion fragment" containing a barnase gene was ligated by using Takara Ligation Kit ver.1 (manufactured by Takara Shuzo).

Introduction into *E. coli* and selection of barnase-active clone

The *E. coli* having the barnase gene introduced thereinto may be selected by, for example, the following method. Since mRNA is degraded in cells by the barnase activity, the synthesis of protein is suppressed and, in its turn, the growth of *E. coli* is inhibited. Thus, the *E. coli* transformed by the barnase gene can be selected by taking advantage of the fact that the desired transformant colony has a smaller size than the *E. coli* colony transformed by the control plasmid free from the mutant barnase gene. When the wild type barnase or a mutant barnase still maintaining the activity comparable thereto is integrated into pHM1, *E. coli* cannot form any colony. Thus, clones having a sufficiently weakened barnase activity can be exclusively selected.

Thus, the plasmid having the mutant barnase gene ligated thereto was precipitated from ethanol and then introduced into *E. coli* LE392 strain by the electroporation method with the use of GenePulser (BioRad) in accordance with the manufacture's instruction. Also, the control plasmid free from mutant barnase was introduced into the *E. coli* LE392 strain in the same manner.

Next, these *E. coli* transformants were plated onto an LB agar medium containing tetracycline (25 μg/ml) and incubated at room temperature (25°C) for 72 hours. Then colonies of a smaller size than the colony of *E. coli* having the control plasmid pHM1 introduced thereinto were screened as colonies of the *E. coli* transformants having the mutant barnase transferred thereinto (Table 1, A).

Selection of mutant barnase clone by barstar gene

As described above, barstar is an enzyme acting as an antagonist to barnase. In *E. coli* wherein barstar is expressed, the enzymatic activity of barnase is inhibited and thus the degradation of mRNA, which otherwise takes place in the presence of barnase, can be inhibited. Therefore, if *E. coli* expressing barstar is transformed by a plasmid containing a barnase gene, the growth thereof will not be inhibited. Accordingly, it is expected that the thus obtained *E. coli* transformant will show little difference in growth rate from the transformant constructed by using the control plasmid free from a barnase gene and, therefore, these transformants will show little difference in colony size too.

An *E. coli* transformant which expressed barstar was constructed in the following manner. A barstar gene described in R.W. Hartley, J. Mol. Biol. 202, 913-915:1998 was excised with HindIII and XbaI and ligated in frame to a tac promoter (de Boer et al., Proc. Natl. Acad. Sci. USA 80, 21-25:1983). The gene, together with a chloramphenicol tolerance gene (N.K. Alton and D. Vapnek, Nature 282, 864-

869:1979), was ligated into the defective transposon which lacked the transferase in a vector described in Herrero et al., J. Bacteriol. 172, 6557-6567:1990. Subsequently, the thus obtained plasmid was introduced into *E. coli* MC1061 strain thereby to transfer the transposon, which contained the barstar gene cassette, into the chromosome of the *E. coli*. It was confirmed based on the chloramphenicol tolerance that this *E. coli* was able to maintain the barstar gene cassette in a stable state. Since the *E. coli* MC1061 strain lacked the lacI gene, the tac promoter was continuously active and thus the barstar gene was constitutively expressed.

The plasmid containing the mutant barnase gene as the insertion fragment and the control plasmid free from the same were each introduced into the above *E. coli* transformant by the electroporation method. Then the *E. coli* transformants were plated onto an LB agar medium containing IPTG (1mM) and tetracycline (25 μg/ml) and incubated at 25°C for 72 hours. Among the colonies of the *E. coli* transformed by the mutant barnase gene, a colony being comparable in size to the colony of the *E. coli* having the control plasmid pHM1, which had been simultaneously plate-cultured, was screened and named "#4-31" (Table 1, B).

Table 1: Screened clone and colony size (mm)

	Screened clone	pHM1 (control)
A: *E. coli* LE392*1	1.77 ± 0.33	2.65 ± 0.47
B: *E. coli* with barstar gene expression*2	1.39 ± 0.10	1.55 ± 0.08

*1: Colony size after incubation on the LB agar plate containing 25 μg/mL of tetracycline at 28°C for 48 hours.

*2: Colony size after incubation on the LB agar plate containing 25 μg/mL of tetracycline at 28°C for 24 hours.

Example 2: Determination of nucleotide sequence of mutant barnase gene

From the *E. coli* screened in Example 1, a cloned mutant barnase gene appropriate for the object of the present invention was prepared. First, the clone #4-31 screened in Example 1 was propagated. Next, the mutant barnase gene fragment in the clone #4-31 was excised with KpnI and XbaI and ligated into the KpnI, XbaI-site of pUC119 having been cleaved similarly with KpnI and XbaI. After amplifying the obtained plasmid by using *E. coli*, a reaction was performed by the cycle sequence method with the use of Taq polymerase (Taq Dye Terminator Cycle Sequencing Kit, manufactured by Applied Biosystems Inc.) in accordance with the manufacturer's protocol. Subsequently, the sequence was analyzed with a DNA Sequencer (Model 373A, manufactured by Applied Biosytems Inc.), thereby yielding the sequence represented by SEQ ID NO:3. Compared with the DNA sequence

of the wild type barnase gene, this sequence had an insertion of T at the 15-position from A of the initiation codon ATG and a deletion of A at the 333-position.

Example 3: Construction of male sterile rice by using attenuated mutant barnase gene

The attenuated mutant barnase gene integrated into pUC119 as described above was cleaved with restriction enzymes XbaI and KpnI and thus the plasmid vector pTS431 represented by SEQ ID NO:5 was constructed. Although this plasmid pTS431 differs from the known plasmid pVE108 (PCT International Publication WO92/13956) in the following points, it is substantially equivalent thereto except for the moieties of the anther-specific promoter and the mutant barnase.

(1) In the plasmid vector pTS431 according to the present invention, the barnase gene (SEQ ID NO:1) in the known plasmid pVE108 has been converted into the mutant barnase gene (SEQ ID NO:3).

(2) A tobacco-origin anther specific promoter is used in the known plasmid pVE108, while the anther specific promoter of rice E1 gene (PCT International Publication WO92/13956) is used in the plasmid vector pTS431 according to the present invention.

(3) In the present invention, a 35S3 promoter (EP 0344029) of 1376 bp, which is not used in the plasmid pVE108, is employed upstream of the barnase gene.

(4) In the present invention, a sequence originating in the downstream of Agrobacterium T-DNA gene 7 excised from

pJD884 (PCT International Publication WO93/09218) is employed whereas such a sequence is not used in the plasmid pVE108.

(5) In the present invention, the region corresponding to lacZ has been deleted from the pUC19-origin moiety. In addition a plasmid (pTS172) having the wild type barnase gene integrated therein is represented by SEQ ID NO:4.

The fragment of about 4.5 kbp was excised with a restriction enzyme EcoRI respectively from pTS431 (a plasmid having the mutant barnase gene transferred thereinto; SEQ ID NO:5) and pTS172 (a plasmid having the barnase gene transferred thereinto; SEQ ID NO:4) and inserted into the EcoRI-site of the intermediate vector pSB11 (T. Komari et al., Plant J. 10(1), 165-174:1996), and further, the T-DNA region thereof was integrated into the acceptor vector pSB1 (T. Komari et al., Plant J. 10(1), 165-174:1996) by way of homologous recombination. By using *Agrobacterium tumefaciens* LBA4404 having the obtained recombinant plasmids (pSB1431 and pSB1172 respectively), rice (variety: Asanohikari) was transformed. While the transformation was performed basically in accordance with the method of Hiei et al. (Plant J. 6(2), 271-282:1994), phosphinothricine (concentration: 10 mg/L) was employed for screening transformants since the thus constructed male sterility genes contained bar gene encoding phosphinithricine acetyl transferase as a selective marker. Phosphinothricine facilitates selection of calluses having the gene transferred thereinto.

A comparison of the rice transformant obtained with the use of the wild type barnase gene with the one obtained with the use of the mutant barnase gene indicates that the transfer of the mutant barnase gene gave rise to a remarkable improvement in the transformation efficiency and an increase in the rate of morphologically normal male sterility transformants, as shown in Table 2.

Table 2: Transformation efficiency

	No. of infected calluses	No. of regenerated calluses	No. of PCR-positive lines*1	No. of morphologically normal male sterile lines
pSB1172 (control)	2838	83	52/83	9/52(17.3%)
pSB1431 (present invention)	787	69	43/45*2	27/28*3(96.4%)

*1: detection of barnase gene fraction by PCR.

*2: examined only 45 lines among 69.

*3: examined only 28 lines among 43.

Effects of the Invention

In the present invention, a barnase gene having a weakened effect is constructed via mutation. A male sterility gene comprising the mutant barnase gene can be introduced into a plant to successfully produce a male sterile plant free from any unfavorable characteristic at a high efficiency with the use of a single gene without resort to the use of a barstar gene.

CLAIMS

1. A mutant barnase gene having one or some mutations at least in part of the DNA sequence of barnase gene, wherein said mutant barnase gene is capable, when anther-specifically expressed in a plant, of making said plant substantially male sterile without exerting any substantially disadvantageous effect on the tissues except for the anthers.

2. The gene as claimed in Claim 1, wherein said mutation is a mutation causing frame shift re-coding.

3. A gene which is derived from a DNA sequence encoding the same amino acid sequence as shown in SEQ ID NO:1 by substitution, deletion, insertion or addition of one to several nucleotides in said DNA sequence, wherein said gene is capable of encoding a protein which makes a plant substantially male sterile when expressed anther-specifically in said plant.

4. A gene comprising a DNA sequence which is derived from a DNA sequence encoding the same amino acid sequence as shown in SEQ ID NO:1 by substitution, deletion, insertion or addition of one to several nucleotides in the latter DNA sequence, and a promoter located upstream of said DNA sequence for allowing an anther-specific expression, wherein said gene is capable of making a plant substantially male sterile when introduced into the genome of said plant.

5. A gene represented by SEQ ID NO:3 which encodes a protein capable of making a plant substantially male sterile when expressed anther-specifically in said plant.

6. A gene comprising the sequence represented by SEQ ID NO:3 and a promoter located upstream of said sequence for allowing an anther-specific expression, wherein said gene is capable of making a plant substantially male sterile when introduced into the genome of said plant.

7. A recombinant vector which contains a gene as claimed in any of Claims 1 to 6 and expresses said gene in a host plant.

8. A method of making a plant male sterile which comprises transforming said plant by a mutant barnase gene as claimed in any of Claims 1 to 6 and allowing said mutant barnase gene to be expressed anther-specifically.

9. The method as claimed in Claim 8, wherein said plant is transformed by said mutant barnase gene by integrating said gene into the genome of said plant.

10. A transgenic plant wherein a gene as claimed in any of Claims 1 to 6 has been introduced.

ABSTRACT

A male sterile plant free from any undesirable characteristic is constructed by anther-specifically expressing barnase gene alone. A barnase gene, which has a mutation and thus sustains its activity at a lowered level, is transferred into a plant and anther-specifically expressed, thereby efficiently providing a male sterile transgenic plant.

BIRCH, STEWART, KOLASCH & BIRCH, LLP

COMBINED DECLARATION AND POWER OF ATTORNEY

FOR PATENT AND DESIGN APPLICATIONS

ATTORNEY DOCKET NO.
230-148P

PLEASE NOTE: YOU MUST COMPLETE THE FOLLOWING:

As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated next to my name; that I verily believe that I am the original, first and sole inventor (if only one inventor is named below) or an original, first and joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Insert Title: MUTANT BARNASE GENE AND TRANSGENIC PLANT TRANSFORMED BY SAID GENE

Fill in Appropriate Information - For Use Without Specification Attached:

the specification of which is attached hereto. If not attached hereto,

the specification was filed on _____ as United States Application Number _____; and /or

the specification was filed on August 3, 1999 as PCT International Application Number PCT/JP99/04167; and was amended under PCT Article 19 on _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I do not know and do not believe the same was ever known or used in the United States of America before my or our invention thereof, or patented or described in any printed publication in any country before my or our invention thereof or more than one year prior to this application, that the same was not in public use or on sale in the United States of America more than one year prior to this application, that the invention has not been patented or made the subject of an inventor's certificate issued before the date of this application in any country foreign to the United States of America on an application filed by me or my legal representatives or assigns more than twelve months (six months for designs) prior to this application, and that no application for patent or inventor's certificate on this invention has been filed in any country foreign to the United States of America prior to this application by me or my legal representatives or assigns, except as follows.

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Insert Priority Information: (if appropriate)

Prior Foreign Application(s)

(Number)	(Country)	(Month/Day/Year Filed)	Priority Claimed
220060/1998	Japan	8/4/1998	☒ Yes ☐ No
			☐ Yes ☐ No
			☐ Yes ☐ No
			☐ Yes ☐ No
			☐ Yes ☐ No

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

Insert Provisional Application(s): (if any)

(Application Number)	(Filing Date)

All Foreign Applications, if any, for any Patent or Inventor's Certificate Filed More Than 12 Months (6 Months for Designs) Prior To The Filing Date of This Application:

Insert Requested Information: (if appropriate)

Country	Application No.	Date of Filing (Month/Day/Year)

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Insert Prior U.S. Application(s): (if any)

(Application Number)	(Filing Date)	(Status - patented, pending, abandoned)

I hereby appoint the following attorneys to prosecute this application and/or an international application based on this application and to transact all business in the Patent and Trademark Office connected therewith and in connection with the resulting patent based on instructions received from the entity who first sent the application papers to the attorneys identified below, unless the inventor(s) or assignee provides said attorneys with a written notice to the contrary:

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Andrew D. Meikle	(Reg. No. 32,868)	Marc S. Weiner	(Reg. No. 32,181)
Joe McKinney Muncy	(Reg. No. 32,334)	Andrew F. Reish	(Reg. No. 33,443)
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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full Name of First or Sole Inventor: Insert Name of Inventor Insert Date This Document is Signed

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CITIZENSHIP:

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Residence (City, State & Country):

CITIZENSHIP:

POST OFFICE ADDRESS (Complete Street Address including City, State & Country):

Full Name of Fifth Inventor, if any see above

GIVEN NAME	FAMILY NAME	INVENTOR'S SIGNATURE	DATE*

Residence (City, State & Country):

CITIZENSHIP:

POST OFFICE ADDRESS (Complete Street Address including City, State & Country):

* DATE OF SIGNATURE

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